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More hidden diversity in a cryptic species complex: a new subspecies of *Leptidea sinapis* (Lepidoptera, Pieridae) from Northern Iran

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Abstract

A new subspecies of *Leptidea sinapis* from Northern Iran, discovered by means of DNA barcoding, is described as *Leptidea sinapis tabarestana* **ssp. nov.** The new subspecies is allopatric with respect to other populations of *L. sinapis* and is genetically distinct, appearing as a well-supported sister clade to all other populations in COI-based phylogenetic reconstructions. Details on karyotype, genitalia, ecology and behaviour for the new subspecies are given and a biogeographical speciation scenario is proposed.

Keywords

allopatry, butterflies, DNA barcoding, Palearctic, taxonomy, Wood White

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Introduction

The cryptic diversity within the *Leptidea sinapis* (Linnaeus, 1758) complex progressively came to light in recent history (Réal 1988) with the discovery of differences in genitalic morphology (Lorković 1993) and allozyme markers (Martin et al. 2003) between *L. sinapis* and *L. reali* Reissinger, 1989. It is considered one of the first documented cases of cryptic species in Europe. Since then, numerous studies have revealed a plethora of new information on the mechanisms of speciation within this species complex (Mazel 2005; Bolshakov 2006; Friberg et al. 2008), including the presence of an additional widespread hidden taxon, *L. juvernica* Williams, 1946 (e.g. Dincă et al. 2011, 2013, 2021; Lukhtanov et al. 2011; Šíchová et al. 2015, 2016; Vodă et al. 2015; Shtinkov et al. 2016; Talla et al. 2017, 2019a, b; Leal et al. 2018; Platania et al. 2020; Yoshido et al. 2020; Näsvall et al. 2021). Despite the explosion of interest in this group, many regions of Eurasia where *Leptidea* species occur are still not well sampled or studied. The new subspecies described in this paper was discovered accidentally in the course of a genetic investigation in order to determine whether any of the populations of *L. sinapis* in Iran belong to the related cryptic species *L. juvernica*.

Materials and methods

Fourteen Iranian specimens from various disjunct populations in NW and N Iran were selected ex. coll. A. Naderi (Tehran) and W. ten Hagen (Germany) and their legs were submitted for DNA barcoding. Samples were processed in the Center for Biodiversity Genomics in Guelph, Ontario, Canada using standard protocols and LepF/LepR primers, supplemented by failure-tracking with mini-primers (mLepF and mLepR) (Hajibabaei et al. 2006). Eleven additional samples from Javaherdeh (VLU396-VLU405, RVcoll10C196) sequenced in 2012 were later added to the dataset. The majority of these sequences were full length barcodes (658 bp). An additional specimen from Javaherdeh included later in our analysis (MR ZF 449) was isolated using the Geneaid Blood and Tissue kit and sequenced in the Czech Republic using RON-HCO primers, and thus only partially overlaps (420 bp) with the standard barcode region. Thirty-six new barcode sequences were submitted to GenBank (Accessions OQ359842–OQ359877). In addition to the sequences pertaining to the new taxon, a selection of 80 other samples from previous studies (Lukhtanov et al. 2011; Dincă et al. 2013; Shtinkov et al. 2016) representing various haplotypes of *L. sinapis* and several other species of *Leptidea* was used to conduct the analyses in this study (Suppl. material 1). All records are publicly available in the BOLD dataset "DS-SINIRAN" (https://doi.org/10.5883/DS-SINIRAN).

A Maximum Likelihood (ML) tree was generated with PHYML online (Guindon and Gascuel 2003) using the AIC criterion and 100 bootstrap replicates. The best-fit model selected by PHYML for the combined dataset (GTR + Γ + I) was further corroborated by IQ-TREE (Nguyen et al. 2015), and parameters from this model were used to conduct a Bayesian analysis in MRBAYES 3.2.6 (Ronquist et al. 2012). The MCMC analysis was allowed to run for 10,000,000 generations until stationary was

reached. Convergence of parameters after the exclusion of the burnin phase was tested using TRACER 1.7.1 (Rambaut et al. 2018). Trees were edited using FIGTREE 1.4.4 (Rambaut 2018). Genetic distances were calculated using the Maximum Composite Likelihood model in MEGA 11.0.8 (Tamura et al. 2021). A haplotype diagram only including *L. sinapis*, *L. juvernica* and *L. reali* was constructed in TCS 1.21 (Clement et al. 2000), with a 95% confidence limit for parsimony. Shorter barcode fragments or those with ambiguous bases were excluded from haplotype analyses.

Male genitalia were examined following maceration in 10% potassium hydroxide (KOH) for 15 minutes at 95 °C, dissection and cleaning under a stereomicroscope and storage in tubes with glycerol. Male genitalia were photographed in a thin layer of 30% ethanol (without being pressed under a cover slip), using a Carl Zeiss Stemi 2000-C stereomicroscope equipped with a CMEX PRO-5 DC.5000p digital camera (RV) or a Leica DFC450 digital camera (ZFF). Care was taken to arrange the measured structures parallel to the focal plane of the stereomicroscope in order to minimize the measurement error. Measurements were performed based on digital photographs using the AxioVision software (Carl Zeiss MicroImaging GmbH). Eight specimens were analysed and the dataset was combined with data from Dincă et al. (2011) (135 specimens). We measured three elements of the male genitalia: phallus length (PL), saccus length (SL) and vinculum width (VW), known to be the most informative for differentiating *Leptidea* species (e.g. Dincă et al. 2011; Shtinkov et al. 2016) (Suppl. material 2). Bivariate scatterplots were generated using VW as a size variable (Shtinkov et al. 2016).

Chromosome preparations were made for ten adult males representing the population from Javaherdeh (field codes VLU396-VLU405) and were processed as previously described (Vershinina and Lukhtanov 2010). Briefly, gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C. Metaphase II (MII) and mitotic plates were examined using the original two-phase method of chromosome analysis (Lukhtanov et al. 2020a). Abbreviation "ca" (circa) means that the count was made with approximation due to overlapping of some chromosomes or due to difficulties in distinguishing between chromosome bivalents and trivalents. Images were edited in open source software GIMP 2.10.32 (The GIMP Development Team 2019) and Inkscape X11 (Inkscape Project 2020). Map was created using Simplemappr (Shorthouse 2010).

Results

None of the barcoded Iranian specimens belonged to *L. juvernica*. Specimens from the Iranian province of East Azerbaijan (Arasbaran) showed several haplotypes identical to those of the common and widespread Eurasian *L. sinapis*; however, samples collected across the Alborz mountains from Talesh to NE Iran represented a unique and well-supported COI clade that appeared as sister to a weakly-supported clade containing all other *L. sinapis* (Figs 1, 2). A comparison of average uncorrected pairwise

Leptidea duponcheli JF512572 Iran Alborz 10km W Ghachsar
Leptidea duponcheli LOWA-AR-21 Armenia Khosrov Nature Reserve
Leptidea duponcheli RVcoll10C240 Iran Hamadan Avaj
Leptidea duponcheli JF512569 France
Leptidea duponcheli ARPI-505a-001 Iran E Azerbaijan Arasbaran
Leptidea lactea JF512717 China
Leptidea lactea JF512719 China
Leptidea lactea JF512718 China
Leptidea morsei JF512618 Kazakhstan
Leptidea morsei HQ004591 Romania
Leptidea morsei CSG08935 Russia Buryatiya Republic
Leptidea morsei GU372563 South Korea Gangwon
Leptidea amurensis JF512620 Mongolia
Leptidea amurensis FJ663713 Russia Altayskiy Kray
Leptidea amurensis JF512622 China
Leptidea amurensis NC 022686 China
Leptidea amurensis FJ663710 Russia Buryatiya Republic
Leptidea amurensis KR363164 Japan
Leptidea amurensis KR363157 Japan
Leptidea juvernica JF512716 Ireland
Leptidea juvernica GU655014 Germany
Leptidea juvernica KC865982 Kazakhstan
Leptidea juvernica EF599643 Slovenia
Leptidea juvernica EF599645 Slovenia
Leptidea juvernica EF599640 Slovenia
Leptidea juvernica JF512651 France
Leptidea juvernica KC866126 Sweden
Leptidea juvernica KC865949 Italy
Leptidea juvernica KC865980 Kazakhstan
Leptidea juvernica HG969220 Russia Novosibirskaya Oblast
Leptidea juvernica KU355288 Bulgaria
Leptidea juvernica JF512648 Kazakhstan
Leptidea juvernica HQ004596 Romania
Leptidea juvernica JF512649 Russia
Leptidea reali JF512704 Italy
Leptidea reali JF512617 Spain
Leptidea reali GU676645 Spain
Leptidea reali KC866117 Spain
Leptidea reali JF512603 Spain
Leptidea reali JF512616 Italy
Leptidea reali JF512712 Italy
Leptidea sinapis ARPI-330d-001 388bp Iran Mazandaran Kojour
Leptidea sinapis DNAwthLeptidea002 Iran Ardabil-Astara Talysh Mts
Leptidea sinapis ARPI-112j-001 611bp Iran Golestan Golestan forest
Leptidea sinapis DNAwthLeptidea003 Iran Ardabil-Astara Talysh Mts
Leptidea sinapis ARPI-408-001 Iran Tehran Laloon
Leptidea sinapis RVcoll10C196 Iran Mazandaran Javaherdeh
Leptidea sinapis VLU396-VLU405 Iran Mazandaran Javaherdeh10 samples
Leptidea sinapis ARPI-524b-001 Iran Gilan Damash
Leptidea sinapis MR_ZF_449 420bp Iran Mazandaran Pol-e Zanguleh
Leptidea sinapis DNAwthLeptidea009 Iran Mazandaran Mt. Samamus
Leptidea sinapis DNAwthLeptidea001 Iran Ardabil-Astara Talysh Mts
Leptidea sinapis DNAwthLeptidea008 Iran Mazandaran Mt. Samamus
Leptidea sinapis MT260486 Bulgaria
Leptidea sinapis MW502826 Spain
Leptidea sinapis KC866088 Bulgaria
Leptidea sinapis DNAwthLeptidea012 Armenia W Dilijan
Leptidea sinapis JF512597 Italy
Leptidea sinapis GU688515 Germany
Leptidea sinapis DNAwthLeptidea006 Iran E Azarbaijan Arasbaran
Leptidea sinapis MW502768 Spain
Leptidea sinapis KC866005 Corsica
Leptidea sinapis KC865994 Sardinia
Leptidea sinapis KC866104 Macedonia
Leptidea sinapis JF513025 Kazakhstan
Leptidea sinapis JF513027 Kazakhstan
Leptidea sinapis KC866098 Macedonia
Leptidea sinapis RVcoll11H081 Germany
Leptidea sinapis HM393183 Austria
Leptidea sinapis KC866101 Czech Republic
Leptidea sinapis JF512697 Czech Republic
Leptidea sinapis JF512592 Romania
Leptidea sinapis HG969226 Russia Novosibirskaya Oblast
Leptidea sinapis JF513026 Romania
Leptidea sinapis KC866102 Sweden
Leptidea sinapis HG969225 Russia Novosibirskaya Oblast
Leptidea sinapis GU688533 Germany
Leptidea sinapis RVcoll17E601 Spain
Leptidea sinapis JF513034 France
Leptidea sinapis KP871088 Spain
Leptidea sinapis DNAwthLeptidea007 Georgia W Tiblisi
Leptidea sinapis JF512693 Bulgaria
Leptidea sinapis DNAwthLeptidea010 Daghestan
Leptidea sinapis ARPI-456d-001 Iran E Azarbaijan Arasbaran
Leptidea sinapis ARPI-400f-001 Iran E Azarbaijan Arasbaran
Leptidea sinapis JF513047 Kazakhstan
Leptidea sinapis DNAwthLeptidea011 Daghestan
Leptidea sinapis KC866100 Spain
Leptidea sinapis MN139254 Italy
Leptidea sinapis KC866089 Italy
Leptidea sinapis GU675913 Spain
Leptidea sinapis JF513046 Kazakhstan
Leptidea sinapis DNAwthLeptidea005 Iran E Azarbaijan Arasbaran
Leptidea sinapis DNAwthLeptidea004 Iran E Azarbaijan Arasbaran
Leptidea sinapis GU675857 Spain
Leptidea sinapis KC866082 Spain
Leptidea sinapis KC866097 France
1/99
0.89/57
1/98
1/100
0.96/98
1/99
0.99/86
1/98
1/97
0.68/51
0.9/61
0.94/85
0.85/66
0.88/77
0.69/61
0.87/81
1/85
0.97/61
0.63/43
0.54/51
0.02
L. sinapis tabarestana ssp. n.
L. sinapis s. str.

Figure 1. Bayesian phylogeny of *Leptidea* COI barcodes. Node support values (Bayesian Posterior Probebilities / ML bootstrap) are shown only for supported nodes. All sequences are 658 bp in length unless indicated otherwise.

distances between this new lineage and other *Leptidea* species showed that it is indeed genetically closer to *L. sinapis* (average: 0.74%; range: 0.42%–1.76%) and further from all the other *Leptidea* (Table 1).

The genitalia of the eight specimens analysed belonging to the above-mentioned COI lineage showed broad overlap with other specimens of *L. sinapis* and a certain degree of variability, despite their fairly restricted geographic origin (Fig. 3). Based on the three characters measured (PL, SL, VW), the male genitalia also indicated a close similarity to *L. sinapis*, with respect to which we did not notice any significant differences.

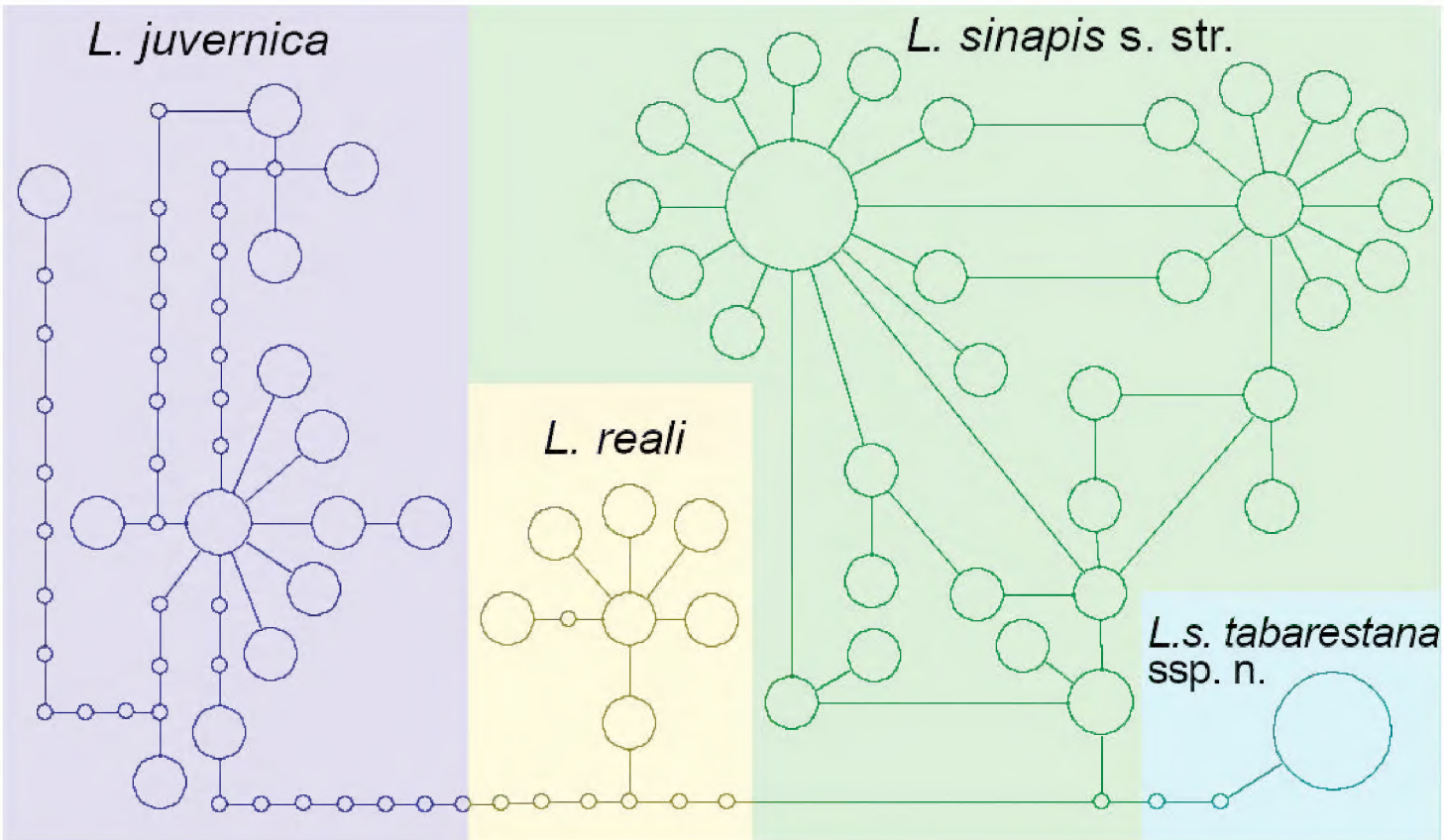

Figure 2. TCS haplotype network for *L. sinapis*, *L. reali* and *L. juvernica*.

Table 1. Average uncorrected *p*-distances (in % of the COI barcoding region) and standard deviation between *Leptidea* taxa.

	L. duponcheli	***L. lactea***	***L. morsei***	***L. amurensis***	***L. juvernica***	***L. reali***	***L. s. sinapis***	***L. s. tabarestana***
L. duponcheli (n=5)	0.27 ± 0.13							
L. lactea (n=3)	5.80 ± 0.13	0.00 ± 0.00						
L. morsei (n=4)	5.94 ± 0.26	2.33 ± 0.26	0.71 ± 0.44					
L. amurensis (n=7)	7.40 ± 0.14	4.23 ± 0.09	3.75 ± 0.13	0.26 ± 0.16				
L. juvernica (n=15)	6.29 ± 0.20	2.51 ± 0.16	3.39 ± 0.24	3.97 ± 0.15	0.30 ± 0.13			
L. reali (n=7)	5.35 ± 0.16	2.33 ± 0.13	2.96 ± 0.25	3.79 ± 0.15	1.75 ± 0.16	0.21 ± 0.07		
L. s. sinapis (n=44)	5.72 ± 0.18	2.71 ± 0.18	3.05 ± 0.21	3.74 ± 0.21	1.97 ± 0.21	0.92 ± 0.15	0.24 ± 0.11	
L. s. tabarestana (n=21)	5.69 ± 0.18	2.76 ± 0.16	2.78 ± 0.25	4.02 ± 0.13	2.00 ± 0.17	0.96 ± 0.19	**0.74 ± 0.20**	0.02 ± 0.05

Considering the allopatric distribution of the new taxon with respect to *L. sinapis*, its similar genitalia, and the fact that the new taxon appears to be genetically closer and phylogenetically sister to the rest of *L. sinapis* specimens, here we describe it as a new subspecies of *L. sinapis*:

***Leptidea sinapis* ssp. *tabarestana* Nazari, Lukhtanov et Naderi, ssp. nov.**
https://zoobank.org/BED12A6B-C1D3-4897-8D40-A955333D6C7C
Fig. 4a–i

Type material. ***Holotype.*** ♂ [white label] "330d= Mazandaran- E Kojour-/Kodir – 1000 m – 2.Jul.[20]10- / leg. A.R. Naderi"; [red label] "Holotype/ *Leptidea sinapis tabarestana* / Nazari, Lukhtanov & Naderi 2023". BOLD Sample ID: ARPI-330d-001;

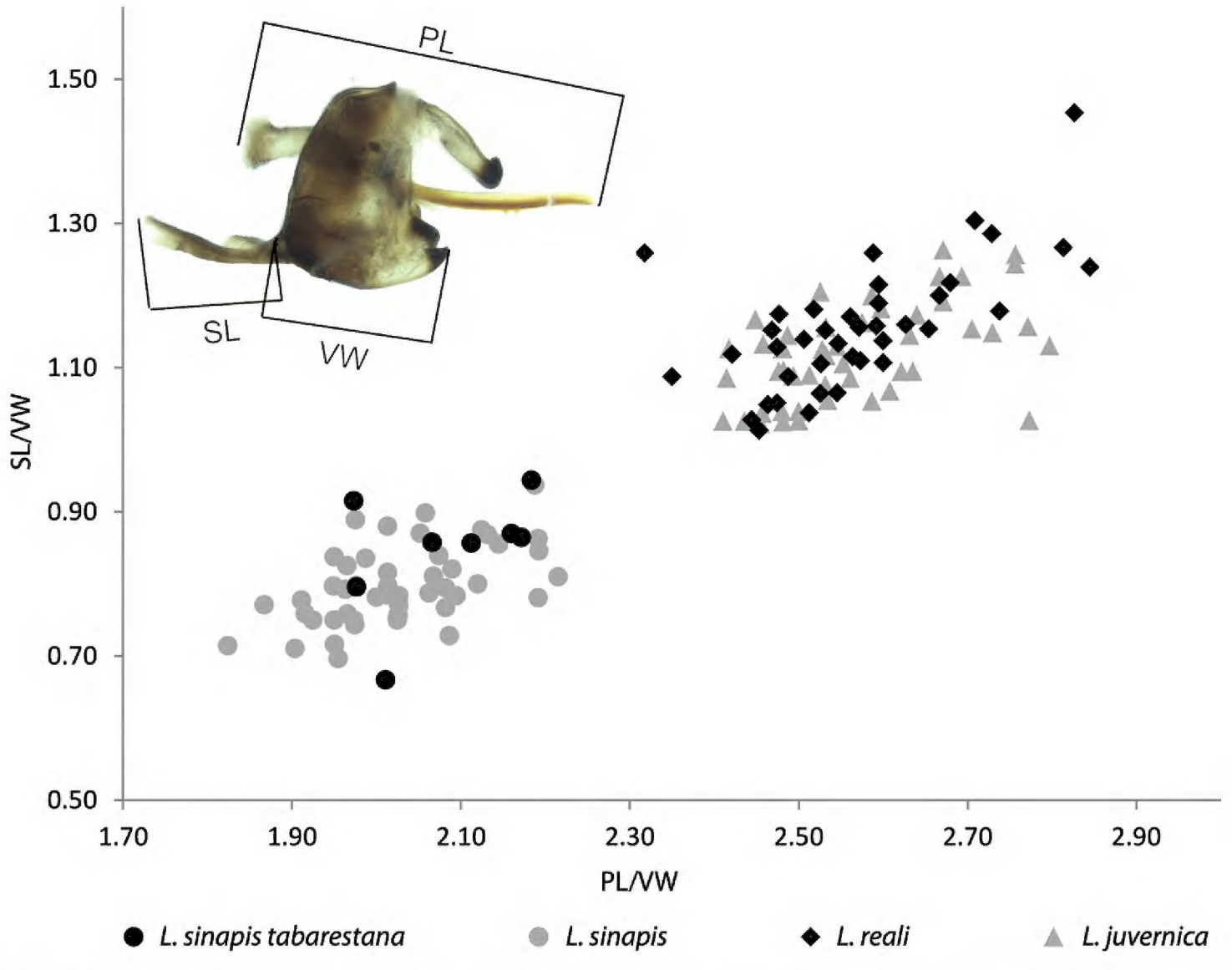

Figure 3. Bivariate scatterplot based on male genitalia morphometry (phallus length, PL; saccus length, SL), using vinculum width (VW) as a size variable. *L. s. tabarestana* ssp. nov. overlaps broadly with *L. s. sinapis* s. str., however it is distinct from *L. juvernica* and *L. reali.* Inset: Male genitalia of *L. sinapis tabarestana* ssp. nov. (specimen MR ZF 449), showing the variables measured.

Deposited in coll. National Natural History Museum & Genetic Resources of Department of Environment, Tehran, Iran.

Paratypes. **Gilan:** 1♂ Damash, 1200m, 26.III.2021, leg. et coll. A.R. Naderi (ARPI-524b-001, AR# 254); 1♀ Khoshkab, rd. Siyahkal-Deylaman, 02.VII.1990, leg. et coll. Harandi. **Ardabil/Gilan:** 2♂♂ 1♀ Paß Ardabil-Astara (Paßhöhe, W Tunnel), 1600m, 10.V.2010, W. ten Hagen. **Tehran:** 1♀ Laloon, 2000–2200 m, 30.VIII.2013, leg. et coll. A.R. Naderi (ARPI-408-001, AR# 186). **Mazandaran:** 1♂ Chalus road, Yush road, 40 km from Pole Zangooleh, 2400 m, 4.VII.1997, leg. & coll. A.R. Naderi (AR# 58); 2♂♂ Galanderoud, 1000 m, 13.VII.07, leg. & coll. A.R. Naderi; 1♂ Siahkal, 03.VII.1990, leg. et coll. Harandi; 1♂ Pol-e Zanguleh – Baladeh Rd, W of Minak, 36.2254°N, 51.58409°E, 15.V.2016, leg. & coll. Z. F. Fric, Biology Centre CAS, Institute of Entomology (IECA) (MR ZF 449); 10♂♂ Javaherdeh (Jirkooh), 36.866, 50.506, 24.VII.2011, leg. V. Lukhtanov & N. Shapoval, in Institut de Biologia Evolutiva (CSIC-UPF), Butterfly Diversity and Evolution Lab (VLU396-VLU405); 43♂♂, 12♀♀ *ibid*, in coll. Zoological Institute of Russian Academy of Sciences;

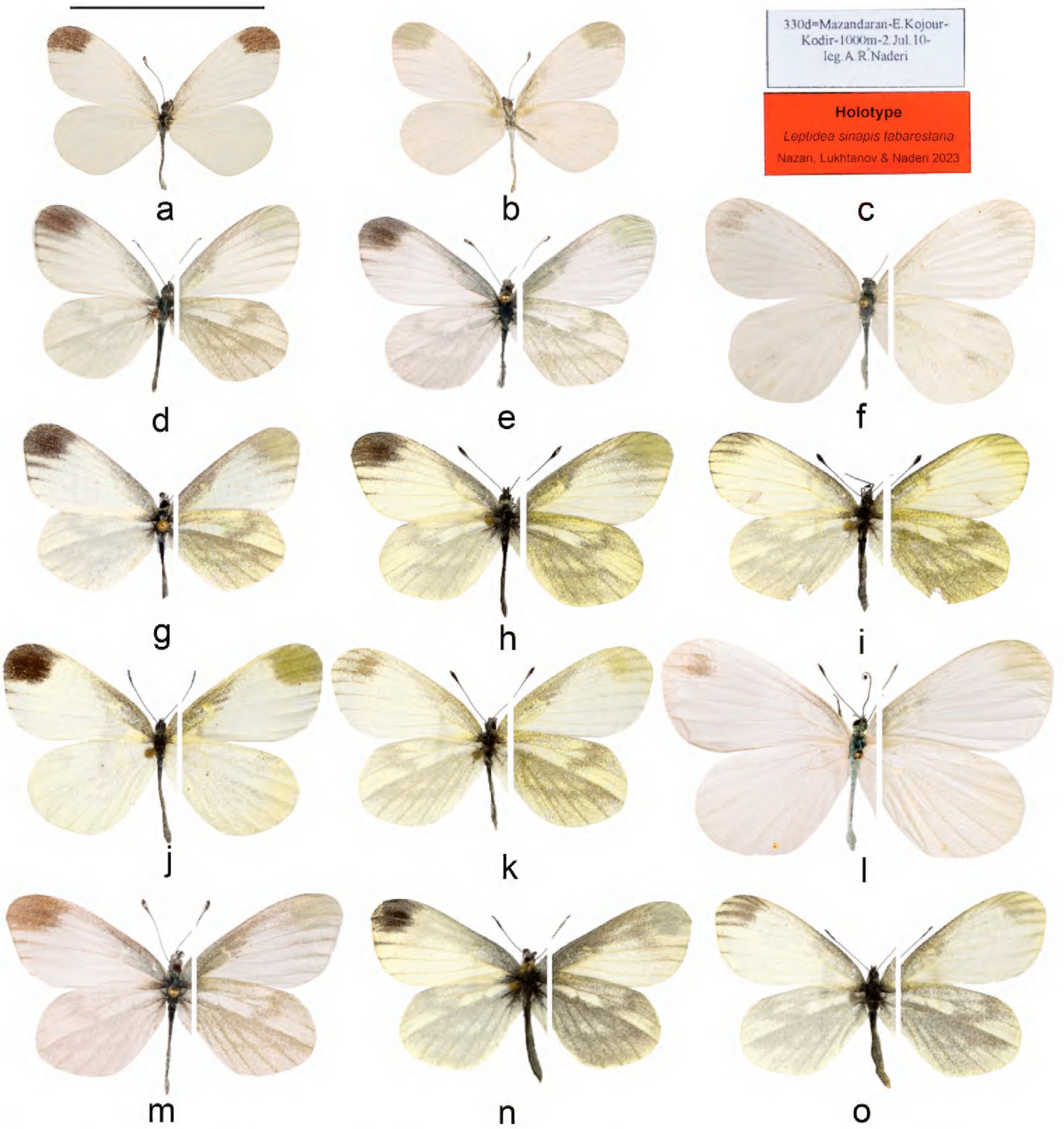

Figure 4. Adults **a–i** *L. sinapis* ssp. *tabarestana* **j–o** *L. sinapis* ssp. *sinapis* **a–c** holotype Mazandaran: Kojur (♂ ARPI-330d-001) **d** Golestan: Golestan forest (♂ ARPI-112j-001) **e** Gilan: Damash (♂ ARPI-524b-001) **f** Tehran: Laloon (♀ ARPI-408-001) **g** Mazandaran: Javaherdeh (♂ MR ZF 449) **h, i** Ardabil/Gilan: Talesh (♂♀ DNAwthLeptidea001–2) **j, k** Iran: E. Azerbaijan prov.: Kaleybar (♂ DNAwthLeptidea006, ♀-004) **l, m** Iran: E. Azerbaijan prov.: Arasbaran (♀ ARPI-479a [not barcoded], ♂ ARPI-456d-001) **n, o** Russia: Daghestan Republic (♂♀ DNAwthLeptidea010-11). Scale bar: 20 mm.

1♂, Javaherdeh (Samamus Mt.), 14.VIII.2010, leg. V.V. Tshikolovets, in Institut de Biologia Evolutiva (CSIC-UPF), Butterfly Diversity and Evolution Lab (RV-coll10C196); 1♂ Samamus Mt., 15 km S Ramsar, 1350 m, 8.VIII.2003, leg. W. ten Hagen; 1♂ Samamus Mt., S Rudbar, N Javaherdeh, 1500 m, 21.VI.2006, leg. W. ten Hagen; 1♂ Samamus Mt., 2800 m, 29.V.2009, leg. et coll. Harandi. **Golestan:** 1♂ Golestan Forest, 800–1000 m, 13.V.2001, leg. & coll. A.R. Naderi (112j, AR# 185).

Description. Male (Fig. 4a, b, d, e, g, h). Length of forewing 16–21 mm; ground colour pure white. ***First generation*** forewing upperside with a rectangular grey-black apical patch, veins v3 and v4 under this patch often covered with dark scales near the outer margin; forewing discal cell covered in grey scales that extend faintly along the costa towards the apex; a small dark patch near the base at the Inner margin. Hindwing upperside veins near the base of the wing covered with dark scales, otherwise without any other markings; the dark scales of the underneath show through. Forewing underside ground colour white with light yellowish-greenish tint at the apex, along the costa and at the discal cell except for a yellowish discoidal spot not covered in grey scales; all veins except v2 covered with dark scales at the outer half of the wing. Hindwing underside ground colour greenish-yellow covered in sparse grey scales; discal cell and space s5 lighter and covered in fewer dark scales; a faint postdiscal band broken into two sections: a costal S- shaped part and a lower postdiscal section in the form of a slightly curved streak. ***Second generation*** similar but grey scales on the underside highly reduced, sometimes completely absent.

Female (Fig. 4f, i). Length of forewing 19–23 mm; similar to male but bigger, forewing apex more rounded; apical dark patch highly reduced, sometimes absent.

Male genitalia (Fig. 3 inset). Based on the eight dissections examined, the male genitalia appear similar to that of the nominotypical *sinapis*. The three elements of the male genitalia (phallus length, saccus length and vinculum width) measured for *L. s. tabarestana* ssp. nov. (PL: 1.47±0.07, SL: 0.60±0.06, VW: 0.71±0.04, n=8) were comparable to those of the nominotypical *L. sinapis* (PL: 1.60±0.08, SL: 0.63±0.04, VW: 0.79±0.05, n=48) (Suppl. material 2).

Diagnosis. Morphologically inseparable from the nominotypical *L. sinapis*, however the new taxon is distinguishable from it only by COI barcodes. Unlike ssp. *sinapis*, which in Iran (East Azerbaijan province) is strictly limited to humid and damp forests or clearings, the new subspecies is found primarily in semi-humid or even semi-dry mountainous habitats.

Etymology. The subspecies name is a reference to "Tabarestan", the medieval name for the mountainous regions south of the Caspian coast in northern Iran and roughly corresponding to the modern-day province of Mazandaran, the type locality of *L. s. tabarestana* ssp. nov.

DNA barcode analysis. The COI barcodes of *L. s. tabarestana* ssp. nov. fall within the Barcode Identification Number (BIN) of *L. sinapis* (BOLD:AAA6298), however they form a unique and distinct cluster that is on average 0.74% (range: 0.42%–1.76%) distant from all other *L. sinapis* (Fig. 1). Uncorrected *p*-distances are smaller than those between *L. sinapis* and *L. reali* (0.92%) or between *L. sinapis* and *L. juvernica* (1.97%) (Table 1). Since the topology of ML and Bayesian trees were similar, only the Bayesian tree is shown with ML bootstrap values plotted on the supported nodes. In both trees, the *L. s. tabarestana* ssp. nov. clade appeared as sister to all other *L. sinapis* samples with strong support (Fig. 1).

Karyotype. Of the 10 specimens studied, only two samples demonstrated metaphase plates suitable for counting the number of chromosomes. Such a low proportion of adult males with dividing cells is a common phenomenon in the genus *Leptidea* and has been noted previously (Lukhtanov et al. 2011). In the sample VLU396, in mitotic cells, the diploid number of chromosomes was determined to be approximately

2n=ca 58. An exact diploid number could not be determined due to numerous overlaps or contacts of chromosomes (Fig. 5).

The MI metaphase cells were not found in the studied individuals; however, MII metaphase plates were found in the sample VLU405. The MII plates demonstrated clear traces of the phenomenon for which we previously used the term *inverted meiosis* (Lukhtanov et al. 2018; 2020a, b). In this type of meiosis, heterozygosity for chromosomal fusions/fissions leads to the very specific chromosomal structures at the MII stage, when heterozygotes retain a configuration similar to that of trivalents. Such trivalent-like structures were observed at the MII stage in the sample U405 (shown in green in Fig. 5d). The number of such trivalent-like structures reached 7, while the total number of chromosome entities was n = 29. If these elements are interpreted as trivalents, then the diploid number can be estimated as 2n = 65. If these elements are bivalents, then the diploid chromosome number is 2n = 58. Thus, the preliminary haploid number of chromosomes can be estimated as n = 29–33.

Previously, a chromosome cline was found in *L. sinapis*, within which the diploid chromosome number gradually decreases from 2n = 106 in Spain to 2n = 56 in Sweden and in eastern Kazakhstan (Lukhtanov et al. 2011, 2018). Thus, the studied population from Mazandaran, Iran has an oriental variant of karyotype, that is, with a relatively low number of chromosomes. We were not able to study the karyotype from the Iranian Talesh; however, the karyotype of the population from Yardimli in Republic of Azerbaijan's Talysh region was studied previously (Lukhtanov 1992). The latter population (Azerbaijani Talysh) demonstrated variation in the haploid chromosome number from n = 28 to n = 34 (Lukhtanov 1992), thus, similar to the Mazandaran population.

Distribution and ecology. So far, the presence of *L. s. tabarestana* ssp. nov. has been confirmed by DNA evidence only in northern Iran, in provinces of Ardabil, Gilan, Mazandaran, Tehran and Golestan (Fig. 6). Specimens from the Talysh mountains in Republic of Azerbaijan, across the border from Iranian Talesh region, show the same karyotype and possibly belong to ssp. *tabarestana*, however this remains to be further confirmed by DNA sequencing. In Turkmenistan, even though reports of *L. sinapis* from the Kopet Dagh mountains are as of yet unconfirmed (Tshikolovets 1998), these also likely belong to ssp. *tabarestana*.

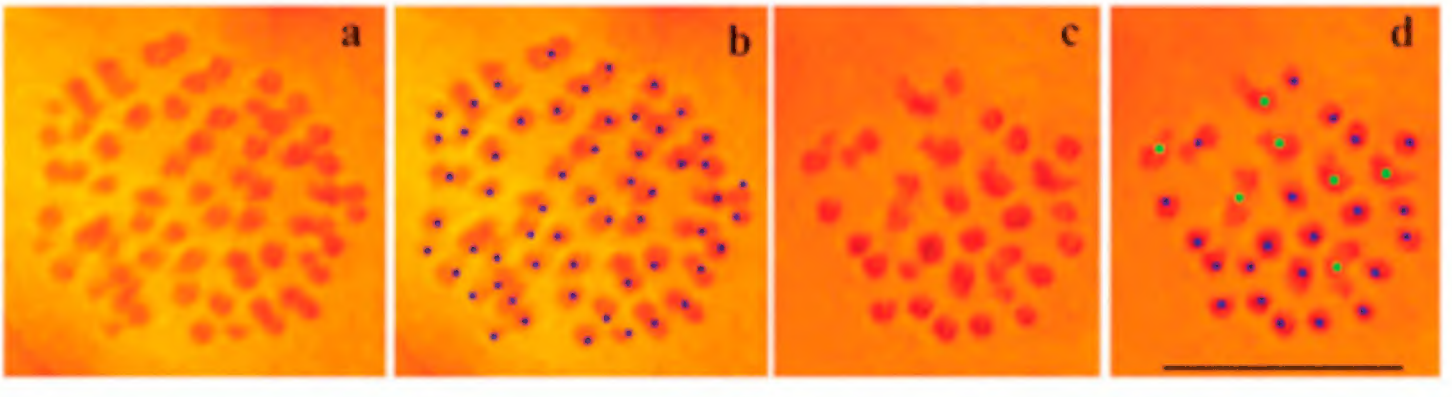

Figure 5. Karyotype of *Leptidea sinapis tabarestana* ssp. nov. **a, b** mitotic cell demonstrating ca 58 chromosomes (sample VLU396) **c, d** MII plate demonstrating 29 entities, 22 entities were interpreted as bivalents (shown by blue dots on Fig. 5d) and 7 entities were interpreted as trivalents (shown by green dots on Fig. 4d). Scale bar: 10 μm.

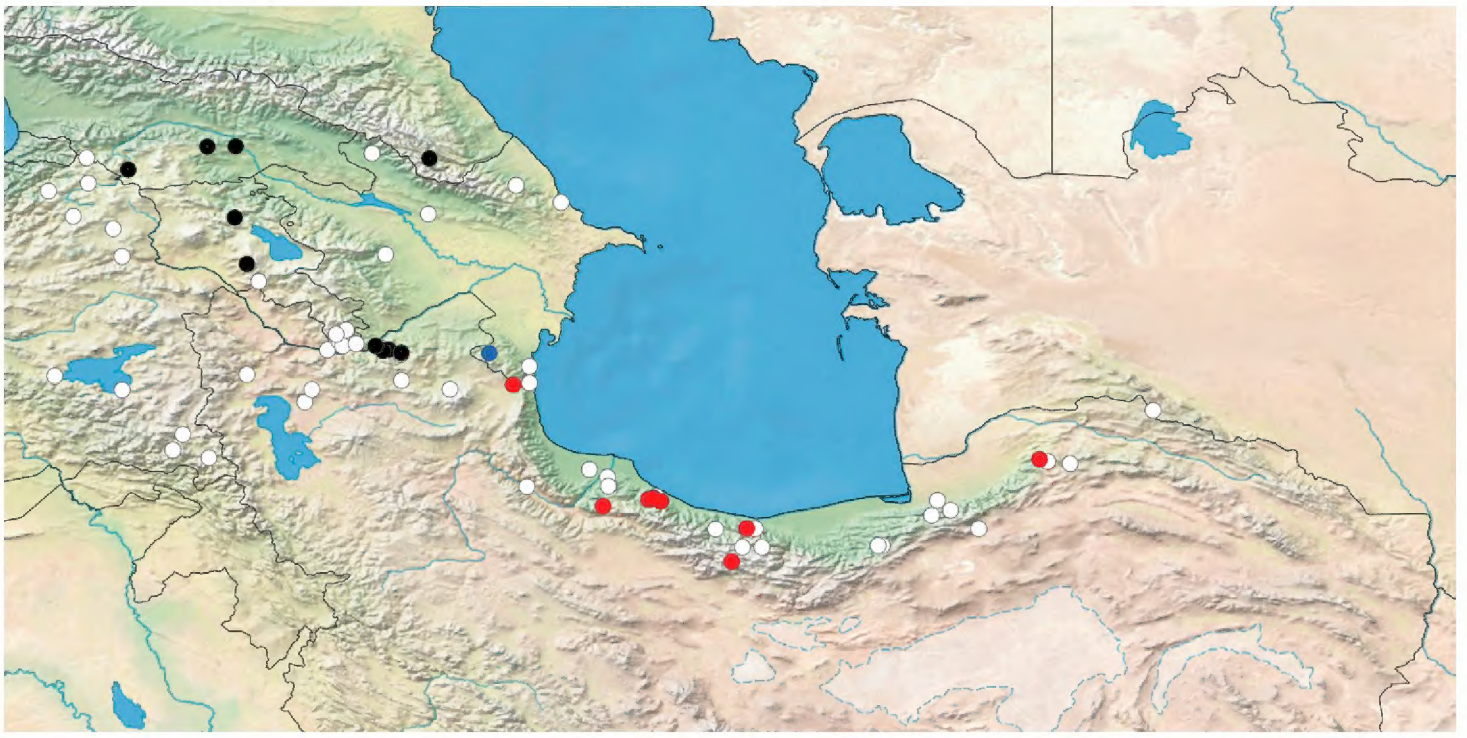

Figure 6. Distribution of *Leptidea sinapis* in E Turkey, S Caucasus and N Iran. Black dots: barcoded *L. s. sinapis*; red dots: barcoded *L. s. tabarestana* ssp. nov.; blue dot: karyotyped sample from Yardamli in Republic of Azerbaijan's Talysh region (most likely *L. s. tabarestana* ssp. nov.); white dots: non-barcoded material, data obtained from literature or personal collections.

In the Iranian Talesh mountains, *L. s. tabarestana* ssp. nov. occurs approximately 100 km from the closest population of the nominotypical *L. sinapis* in Arasbaran region. The habitat of ssp. *tabarestana* is in the Alborz forest belt, in humid meadows, forest river banks, forest clearings, and sometimes gardens at mountain steppes from 1000 to 2000 m above sea level. Adults fly mostly in undisturbed or lightly-grazed habitats with lush of green vegetation (Fig. 7). The accompanying species include *Ochlodes hyrcana* (Christoph, 1893), *Pieris napi mazandarana* Eitschberger, 1987, *Lasiommata adrastiodes* (Bienert, [1870]), and *Maniola jurtina* (Linnaeus, 1758). It is normally found in two (or maybe three) generations, from April at lower altitudes to the end of September at higher altitudes. The early stages of *L. s. tabarestana* ssp. nov. are unknown, however adults are often seen near *Lathyrus* plants (AN, personal observation). Even though the larval host plant is likely among the herbaceous Fabaceae of the genera *Lathyrus*, *Vicia*, *Lotus* etc., it is as of yet unrecorded and thus it is unclear if ssp. *tabarestana* displays any host plant preferences different from the rest of populations of ssp. *sinapis*.

Discussion

Reissinger (1989) recognized twelve subspecies of *L. sinapis* across its range, including *reali* and *juvernica*, both of which were later confirmed as separate species (Dincă et al. 2011, 2013). Since then, this complex has taken a central stage in efforts to understand the mechanisms of cryptic speciation in butterflies, and thus the idea of the existence of subspecies within *L. sinapis* seems to have slowly faded away. Modern taxonomic treatments of the group (e.g., Bozano et al. 2016) regard all populations of *L. sinapis*

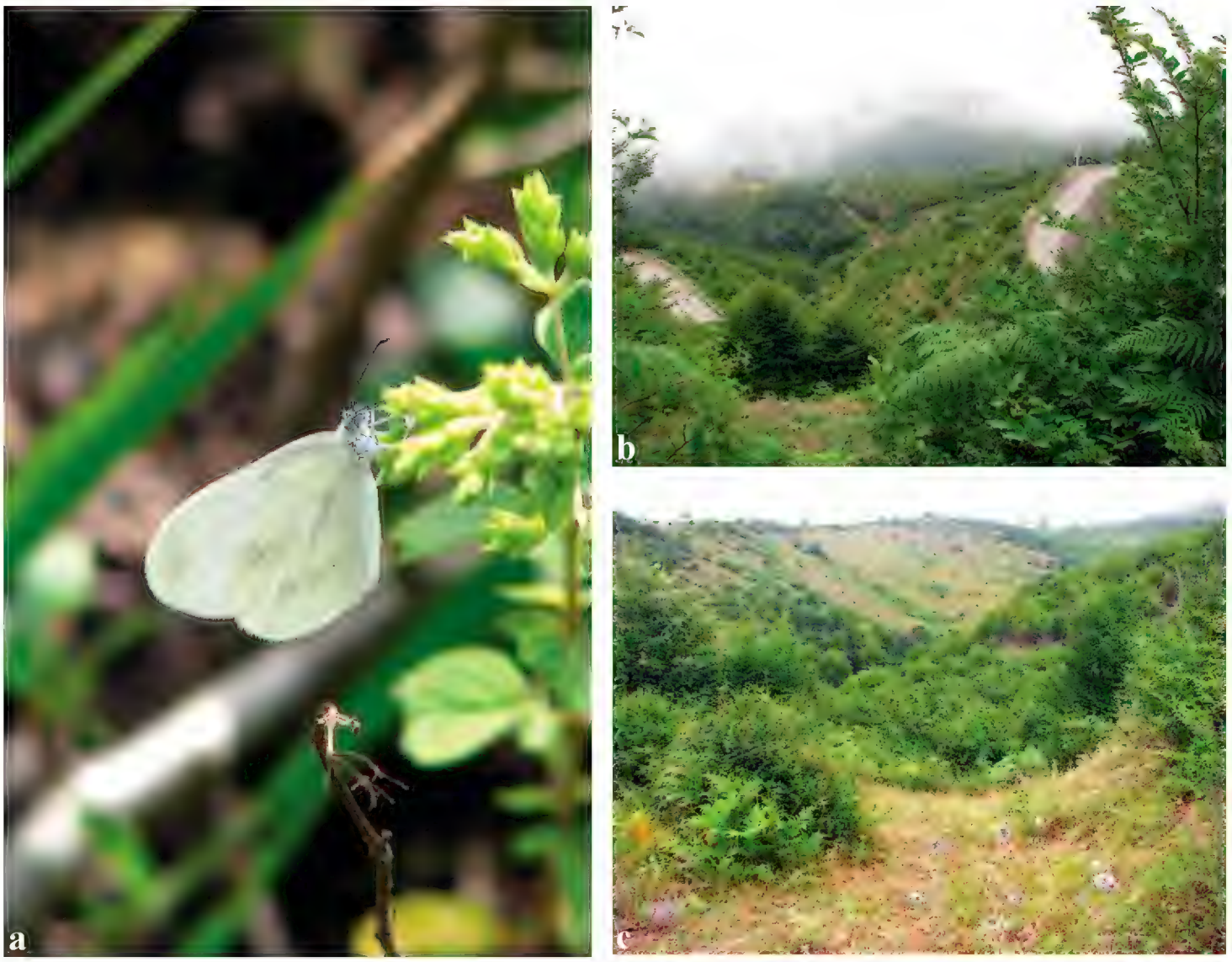

Figure 7. *Leptidea sinapis tabarestana* ssp. nov. **a** adult **b, c** habitat in Iran, Mazandaran Prov., Javaherdeh (Jirkooh), 24.VII.2011. Photos: V. Lukhtanov.

from Europe to NW Mongolia as a single entity, corresponding to the nominotypical subspecies. It occasionally flies in sympatry with closely related and extremely similar species of *Leptidea* across its range and can be separated from them only by DNA sequencing and analysis of karyotype or genitalia.

In a similar vein, in this study we found no single external morphological character or combination of characters that could reliably separate *L. s. tabarestana* ssp. nov. from the nominotypical *L. sinapis*. Individual variation in morphology observed within *L. s. tabarestana* ssp. nov. is not unexpected, as similar variation can also be seen in *L. s. sinapis*, as well as other species within the genus. In the Arasbaran mountains in NW Iran, where the nominotypical *L. sinapis* is found, individuals flying in colder slopes at high altitudes (1700–2000 m) tend to be smaller and darker, while those found in warmer forests at lower altitudes (1200–1400 m) are usually larger in size and have a lighter complexion.

Recent studies have estimated the age of the most recent common ancestor (MRCA) of *L. sinapis* at 1.5 mya, and for MRCA of *sinapis+juvernica* at 3 mya (Talla et al. 2017). The subsequent dispersal of *L. sinapis* eastward however appears to have occurred much later, either before or after the Last Glacial Maximum (LGM) (24,000 to 17,000 years ago) (Lukhtanov et al. 2011). During the Pleistocene, dense forests covered the entire northern Iran, from the northwest (Azerbaijan province) across the Alborz mountains

and extending further into the northeast (Kopet Dagh); However, since the Last Glacial Maximum (LGM; 21 kya), the Alborz mountain range has been nearly entirely isolated from all other regions surrounding it. Subsequent decline in forest cover resulted in isolated refugia in parts of southern Caucasus as well as in northern Iran (Yousefi et al. 2015; Asadi et al. 2018; Parvizi et al. 2018; Liu et al. 2019; Saberi-Pirooz et al. 2020). With the likely extinction of intervening populations, the range of many butterflies adapted to this habitat – including the ancestral *L. sinapis* – became fragmented, resulting in the geographic and genetic isolation of *L. s. tabarestana* ssp. nov.

Presence of *Wolbachia* endosymbionts affecting mtDNA in *Leptidea* has been noted previously (e.g. Solovyev et al. 2015) and we cannot rule out that this may have had an effect on our results. Further studies are needed to confirm the presence of *L. s. tabarestana* ssp. nov. in the Republic of Azerbaijan and in Turkmenistan. Potential sympatric occurrence of the two entities in the intervening areas in NW Iran needs to be investigated. If the two are found to co-occur sympatrically and synchronically without geneflow, or other new information (e.g., karyotype, nuDNA, morphology etc.) comes to light that clearly signals the two taxa to be distinct at species level, the taxon *tabarestana* may be raised as bona species.

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Supplementary material 1

Material examined and GenBank accessions

Authors: Vazrick Nazari, Vladimir A. Lukhtanov, Alireza Naderi, Zdenek Faltýnek Fric, Vlad Dincă, Roger Vila

Data type: excel file

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Link: https://doi.org/10.3897/compcytogen.17.102830.suppl1

Supplementary material 2

Length of male genitalia components (phallus, saccus, vinculum) and ratios (PL/VW, SL/VW) among *Leptidea* specimens measured in this study

Authors: Vazrick Nazari, Vladimir A. Lukhtanov, Alireza Naderi, Zdenek Faltýnek Fric, Vlad Dincă, Roger Vila

Data type: excel file

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